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*Marian Christopher*  
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Lynn E. SPITLER  
and Anthony E. Maida III

Serial No.: 09/300,978

Filing Date: 28 April 1999

For: METHODS OF ELICITING AN  
ANTITUMOR IMMUNE RESPONSE  
TO PROSTATE TUMORS (as amended)



Examiner: Phillip Gambel, Ph.D.

Group Art Unit: 1644

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REPLACEMENT BRIEF ON APPEAL

Assistant Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

Appellants hereby appeal from the final rejection of claims 13-24 mailed 27 June 2001. A Notice of Appeal was filed 18 September 2001. An Appeal Brief and a Petition for an Extension of Time for filing the Brief of one month was filed 18 December 2001 ("Original Appeal Brief"). However, Notification of Non-Compliance with 37 CFR § 1.192(c) mailed 12 March 2002, with a one month due date for response, was received and, as such, this Appeal Brief ("Replacement Appeal Brief") is timely filed. Appellants respectfully request that the rejections be reversed. In accordance with 37 C.F.R. § 1.192, this Brief is filed in triplicate.

I. Real Party in Interest

The present application is assigned to Jenner Technologies, a California corporation.

II. Related Appeals and Interferences

Appellants are aware of another appeal with respect to the grandparent application of the present application, US Ser. No. 08/105,444 filed August 11, 1993, which may have a bearing on the Board's decision in the pending Appeal.

III. Status of claims

The application was filed with claims 1-12. All of these claims have been cancelled by the preliminary amendment filed 28 April 1999. Claims 13-24 have been added in that same preliminary amendment. Upon filing of the Original Appeal Brief filed 18 December 2001, the amendment filed 15 August 2001 had been entered which amended claim 13 and canceled claims 14 and 17, according to the Advisory Action mailed 18 September 2001. In addition, the Amendment filed 18 December 2001 with the Original Appeal Brief has been entered. The Notification of Non-Compliance with 37 CFR 1.192(c) indicates that "applicant's amd. after final (after appeal brief), filed 1/18/01 has been entered," however, a telephone conference with Examiner Gambel on 2 April 2002 confirmed that the amendment referenced in the Notification was the Second Amendment Under 37 CFR § 1.116 that was filed 18 December 2001. Pending claims 13, 15-16, and 18-24 are rejected.

IV. Status of Amendments

In response to a final rejection herein, appellants submitted an Amendment filed 15 August 2001 amending claim 13 and canceling claims 14 and 17 as described above. According to the Advisory Action mailed 18 September 2001, this amendment has been entered. Appellants also submitted on 18 December 2001 a Second Amendment in response to final rejection. In this Second Amendment, claims 15, 16, 18, and 19 have been amended to correct

the antecedent basis with respect to claim 13, and claim 13 has been further amended to simplify the claim language. This amendment has been entered as well.

V. Summary of the Invention

Prior art formulations for vaccines designed to produce an antitumor response from an immune system have been based on the use of antigens that are uniquely associated with the tumors *per se*. The present invention represents a different approach in that, rather than such uniquely tumor-associated antigens as active ingredients, the present invention employs antigens, namely prostate-specific membrane antigen (PSMA) and prostatic acid phosphatase (PAP), that are associated with the host prostate tissue, that is, the antigens are found in the prostate in contrast to other tissues. Generally, these antigens are found both in the normal prostate and in malignant prostate tissue. (See page 4, lines 11-22.) The invention takes advantage of the fact that the prostate is not an essential organ and thus an immune response which could include disruption of normal tissue is acceptable. See page 4, lines 11-22.

In the Notification of Non-Compliance, the Examiner invited appellants to address the definition of tumor-associated antigens (TAAs), including whether PSMA and PAP were considered TAAs by the skilled artisan at the time the invention was made. A definition of TAA is provided herewith that was found at the Aventis website ([http://www.aventispasteur.com/us/media/kit\\_cancer4.html](http://www.aventispasteur.com/us/media/kit_cancer4.html)), a copy of which is attached herewith as Exhibit B. This definition states that TAAs are expressed by tumor cells but either not or hardly expressed by normal cells. As such, TAAs characterize tumors but do not characterize normal tissue. In contrast, PSMA and PAP are over-represented in normal prostate and indeed characterize normal tissue. Attached is an abstract (found at <http://gamma.mbb.kj.se/rpap.html>) indicating PAP is found in high concentrations in the prostate. PSMA and PAP may be elevated when prostate tumors are present, but they do not characterize prostate cancer because they are over-represented or found in high concentration in normal prostate. Please see page 9, lines 28-31 of the present specification. In sharp contrast to TAAs which are either not present or barely present in normal tissue, it is clear from the Spitler patent (U.S. Patent No. 5,738,867) cited in the rejection under 35 U.S.C. §103 that the TAAs

contemplated therein were associated with tumors and not normal tissues, such as carcinoembryonic antigen and melanoma associated antigen. Please see col. 4, lines 7-10.

The PAP or PSMA antigen is specifically associated with prostate, whether normal or malignant. The antigen can be supplied, for example, as the antigen *per se* or as an expression system which is able to produce the protein or peptide *in situ* in the subject. The invention is directed to methods of use. (See the paragraph bridging pages 4 and 5.)

Thus, the invention described in claims 13, 15-16, 18-24 is directed to methods of eliciting an antitumor immune response to prostate tumors using PSMA and/or PAP, or a nucleic acid that generates either antigen as an active ingredient. Claims 20-23 are directed to the same method where either the antigen is further encapsulated in a liposome or coupled to a liposome and/or the liposome contains an adjuvant. The method of claim 24 further defines the method as being directed to a subject afflicted with prostate cancer and/or wherein the subject has been surgically treated to excise the tumor but is at risk for recurrence.

## VI. Issues

The following issues are presented for review.

1. Whether structures of nucleotide sequences encoding PSMA and PAP that are known in the art must be provided in the specification if the specification as filed includes reference to such prior knowledge. This issue is reflected in a rejection under 35 U.S.C. § 112, first paragraph (written description).

2. Whether the language in claim 12 of the originally filed application provides written description basis for the identical language as used in new claim 24. This issue is reflected in an additional rejection under 35 U.S.C. § 112, first paragraph (written description).

3. Whether issue is moot with respect to the enabling disclosure for “over-represented prostate specific antigen” and “immunologically effective portion thereof” due to deletion of these phrases from the claims.

4. Whether the cited Spitler *et al.* (U.S. Pat. No. 5,738,867) and Israeli *et al.* (U.S. Pat. No. 5,538,866) references can be used to render the present claims obvious under 35 U.S.C. § 103 and concomitantly ignored for purposes of enablement under 35 U.S.C. § 112, first paragraph. This issue is reflected in the “squeeze” rejection under both 35 U.S.C. § 103 and 35 U.S.C. § 112, first paragraph (enablement).

5. Whether the claimed methods are obvious under 35 U.S.C. § 103 over the combination of Spitler *et al.* (U.S. Pat. No. 5,738,867) in view of Israeli *et al.* (U.S. Pat. No. 5,538,866) and the present disclosure where neither Spitler nor Israeli nor the acknowledged art in the present specification suggests that a normal tissue antigen shared by a tumor be used to elicit an active tumor response.

6. Whether the patented method claims in U.S. Pat. No. 9,925,362 ('362) are a species of the instant methods such that there is double patenting where the instant claims contain no reference to prostate-specific antigen (PSA), and the patented claims are directed solely to methods relating to PSA.

#### VII. Grouping of Claims

The inventive concept of all claims is the same and all claims may be considered together for purposes of the rejection under 35 U.S.C. § 103.

However, it should be evident that the rejection under 35 U.S.C. § 112, as set forth in issue No. 1 above, is inapplicable to claims 15-16, which are limited to the two specific known antigens: prostate-specific membrane antigen (PSMA) or prostatic acid phosphatase (PAP).

#### VIII. Argument

It is believed that issues 1-6 should be resolved in favor of appellants for the following reasons:

- A. The structures of nucleotide sequences encoding PSMA and PAP are known in the art and sufficiently show inventor was in possession of claimed method of using such nucleotide sequences.

It is respectfully submitted that a *prima facie* case of lack of written description under 35 U.S.C. § 112, first paragraph, has not been established for claims 13 and 18-24 as alleged in paragraph numbered “3” of the 27 June 2001 Office Action. A description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the Examiner to rebut the presumption. *In re Marzocchi*, 169 U.S.P.Q. 367 (C.C.P.A. 1991). The Examiner has the initial burden of presenting by a preponderance of evidence why a person of ordinary skill in the art would not recognize in an appellant’s disclosure a description of the invention defined by the claims. Please see, for example, the Interim Guidelines for the Examination of Patent Applications Under 35 U.S.C. 112, paragraph 1 “Written Description” Requirement (“Written Description Guidelines”) § IIA 63 Fed. Reg. 114 (1998) (proposed June 15, 1998). An adequate written description of the invention may be shown by any description of sufficient, relevant, identifying characteristics so long as a person skilled in the art would recognize that the inventor had possession of the claimed invention. Please see Written Description Guidelines § IIC.

With respect to the term “nucleic acid sequences,” claim 13 includes nucleic acid sequences encoding PSMA or PAP *per se*. The structure of these proteins and of the nucleic acids encoding them is known in the art. See page 8 of the specification, lines 1-5 (PAP) and page 9 of the specification, lines 9-11 (PSMA). The specification further describes the nucleic acids of claim 13 on page 6, line 21 to page 7, line 14. In addition, the specification on page 16, line 24 to page 17, line 4, describes how DNA encoding polypeptides such as PAP or PSMA may be administered to a subject by way of a viral expression vector. Hence, the specification demonstrates that appellant plainly had possession of “nucleic acid sequences” as set forth in claim 13.

Moreover, the MPEP § 2173.05(t) states that “A claim to a chemical compound is not indefinite merely because a structure is not presented ...” and “Chemical compounds may be

claimed by a name that adequately describes the material to one of skill in the art.” The known antigens, PSMA and PAP, are adequately described to one of skill in the art. Indeed, the Examiner has cited the Spitler patent (U.S. Pat. No. 5,738,867) that sets forth the nucleotide sequence for PSMA. Other references are cited in the specification that disclose the sequence for PAP. A skilled artisan would therefore understand the description of PSMA and PAP without the need to incorporate the sequences thereof in the application.

Furthermore, claims 15-16 are not rejected for improper written description. Thus, it is evident that claims 15-16, which are directed either to the PSMA (claim 15) or PAP (claim 16) antigen, properly conform to the written description requirement although no polypeptide sequence has been recited in the specification. Similarly, although the nucleotide sequences encoding PSMA or PAP specifically have not been recited in the specification, these nucleotide sequences are known in the art and likewise properly conform to the written description requirement. Thus, the rejection for lack of written description may be withdrawn.

B. The subject matter of claim 24 represents subject matter originally claimed in the application as filed (claim 12).

A *prima facie* case of lack of written description under 35 U.S.C. § 112, first paragraph has not been established with respect to claim 24 as alleged in the first occurrence of the paragraph numbered “8” of the 27 June 2001 Office Action. No reasoning has been set forth by the Examiner that would show that a skilled artisan would not recognize that the inventors had possession of the claimed invention. As discussed above, a description as filed is presumed to be adequate and the initial burden is on the Examiner to make a showing otherwise. The Examiner merely baldly states that the original specification does not provide support for the claim language: “wherein said [sic, subject] is afflicted with metastatic prostate and/or where said subject has been surgically treated to excise said tumor but is at risk for recurrence.”

The subject matter of claim 24 represents subject matter originally claimed in the application as filed (claim 12). Original claim 12 required that the subject be

- (a) afflicted with metastatic prostate cancer; and/or
- (b) been surgically treated to excise the tumor but is at risk for recurrence (with the optional limitation that the subject is in a “neoadjuvant” setting) or
- (c) wherein the subject is a potential prostate tumor-bearing subject.

The changes simply delete the last alternative (c) and the optional limitation to the second (b). Thus, the subject matter is completely disclosed *in haec verba* in the application as originally filed. Accordingly, this basis for rejection may properly be withdrawn.

C. Issues are moot with respect to enablement of “over-represented prostate specific antigens” and “immunologically effective portion thereof”

It is believed that the rejection under 35 U.S.C. § 112, first paragraph (enablement) has been overcome with respect to the language relating to “immunologically effective portion thereof.” This language was deleted in the First Amendment in response to final rejection, which is referenced by the Advisory Action that states that the rejection over “immunologically reactive/effective portion” has been overcome. Although no rejection in the Advisory Action was specified, it is believed that the rejection in question is 35 U.S.C. § 112, first paragraph (enablement), and thus it is believed that there is no issue on appeal with respect to this portion of the rejection.

The phrase “over-represented prostate specific antigens” has been deleted in the Second Amendment Under 37 C.F.R. § 1.116, which has been entered. Further, the Examiner alleges on page 4, paragraph numbered “4” of the Final Office Action mailed 21 June 2000, that “the specification [is] enabling for ‘full-length ... PSMA and PAP.’” As the amended claims are directed only to full-length PSMA and PAP, the rejection under 35 U.S.C. § 112, first paragraph (enablement) may be properly withdrawn because this issue is moot.

D. The squeeze rejection under 35 U.S.C. § 103 and 35 U.S.C. § 112, first paragraph (enablement) is improper as the references cannot be used to render obvious the



claimed invention while being ignored to render the present claims not enabled under 35 U.S.C. § 112, first paragraph.

If the enablement rejection is not overcome by the amendment to claim 13 in the Second Amendment in response to final rejection, the rejection under 35 U.S.C. § 112, first paragraph (enablement) should not stand because an improper “squeeze” rejection has been made. The Office argues both that a) a skilled person would not be enabled at the time the application was filed to use PSMA and PAP, i.e. the “over-represented prostate specific antigens” in the methods of the invention under 35 U.S.C. § 112, first paragraph (enablement), and b) the art references were sufficiently enabled to teach a skilled person to use PSMA and PAP according to the methods of the invention under 35 U.S.C. § 103. Such rejections made concomitantly are inconsistent. Accordingly, this basis for the enablement rejection may properly be withdrawn.

E. The prior art references cited, taken individually or together, fail to suggest the present invention.

As a preliminary matter, if the enablement rejection in section “C” above stands, then the obviousness rejection under 35 U.S.C. §103 should be withdrawn on the basis of the arguments presented in section “D” above. However, assuming that the enablement rejection is withdrawn, it is respectfully submitted that the present invention is not rendered obvious by Spitler in combination with Israeli and in view of the art-acknowledged methods of delivering antigens of interest to stimulate antitumor responses as disclosed on pages 10-19 of the instant specification, as asserted by the Office.

The Office has not established a *prima facie* case of obviousness. According to Section 2143 of the MPEP, a *prima facie* case of obviousness is presented if three requirements are met. First, there must be a suggestion or motivation, either in the references themselves or in the knowledge in the art, to modify the reference or to combine teachings. Second, there must be a reasonable expectation of success. Third, the prior art reference must teach or suggest all the claim limitations.

1. No motivation to combine teaching of antigen not shared by normal prostate cells with teaching of antigen normally produced from prostate cells.

The basis for the rejection appears to reside in the general involvement of antigens characteristic of the prostate in treatment of prostate cancer. The rejection fails to recognize how different the claimed approach is from that taught by the references.' The difference can be succinctly stated once again: (a) an antigen shared by the normal cells of the prostate with prostate tumors (i.e., that are not unique to the tumor and/or are expressed in high concentrations in such normal cells) is used to elicit an active immune response against the tumor, and (b) the antigen is not used as a target for an antibody or immunoconjugate. Appellants are unable to find any suggestion in this combination of references that a normal tissue antigen shared by the tumor, such as PSMA or PAP as claimed, should be used to elicit an active tumor response. The Examiner has been unable to point to any such suggestion.

Appellants certainly recognize that it is the combination of references that has been applied not the references individually; however, it will be helpful to summarize the teachings of each reference.

The primary reference, Spitler, refers to the use of a tumor associated antigen which is not found in normal tissue as the active ingredient in a vaccine. The whole concept of the present invention resides in using, instead of such an obvious target, an antigen that occurs in normal tissue.

Israeli discusses the isolation of nucleic acid encoding PSMA. Also, Israeli discusses *ex vivo* production of antibodies against PSMA and does not suggest eliciting an immune response in a subject. Therefore, Israeli does not cure the defects of Spitler because it also fails to suggest a method for eliciting an immune response in a subject using an antigen that is over-represented in normal tissue or occurs in normal tissue in high concentrations.

The documents cited in the specification on pages 10-19 discuss general methods of delivering antigens of interest to stimulate antitumor responses. However, none of these documents cure the defects of Spitler and Israeli because they fail to discuss methods for eliciting an immune response in a subject using an antigen that is over-represented or occurs in high concentrations in normal tissue.

Eliciting an immune response in a subject using an antigen that is over-represented or occurs in high concentrations in normal tissue is nowhere suggested in the cited art.

The Court of Appeals for the Federal Circuit has stated that there are three possible sources for a motivation to combine documents: the nature of the problem to be solved, the teachings of the prior art, and the knowledge of persons of ordinary skill in the art. *In re Rouffet*, 47 USPQ.2d 1453 (Fed. Cir. 1998).

The asserted motivation for combining the documents ignores the fact that PSMA is not a tumor-associated antigen, *i.e.*, an antigen not associated with normal tissue. Thus, the disclosure of Spitler where an antigen uniquely associated with the tumor and not expressed in normal tissue, and thus foreign to the host, is used as an active ingredient in a vaccine provides no incentive (absent the invention) to combine its teachings with a document which teaches a normally produced antigen. Thus, a *prima facie* case of obviousness has not been established because there is no motivation to modify or combine references.

2. No reasonable expectation of success.

Neither reference suggests forming an immune response against an antigen found in normal prostate tissue and thus neither reference mentions or suggests that such an approach would be successful.

Further, it is respectfully submitted that a skilled artisan would not expect an immune response to be elicited against an antigen found in high quantities in normal tissue. Thus, the appellant's approach and results are unexpected.

For these reasons, the rejection over Spitler in combination with Israeli in view of the present disclosure may properly be withdrawn.

F. The double patenting rejection no longer applies as PSA has been deleted from the claims

Appellant inadvertently omitted a response to the double patenting rejection as kindly pointed out by the Examiner in the Notice of Non-Compliance dated 12 March 2002. When the double patenting rejection was made, as well as when it was made final in the 27 June 2001 Office action in the second occurrence of the paragraph numbered "7," the claims were directed to methods relating to PSA as well as PSMA and PAP. Since this final rejection, the claims have

been amended, and the amendments have been entered, to delete reference to PSA. The patent cited in the double patenting rejection is directed only to methods relating to PSA, and thus it is respectfully submitted that this rejection is not applicable to the amended claims. Withdrawal of this rejection properly may be made.

#### IX. Appendices

Attached hereto is Appendix A containing a copy of the claims involved in the Appeal. Attached Appendix B contains a definition of TAA. Appendix C is attached as a further showing that a skilled person would understand that PAP is found in high concentrations in normal prostate.

#### Conclusion

For the reasons stated above, appellants respectfully request that the rejections under 35 U.S.C. § 112, first paragraph for asserted lack of written description and enablement as well as the rejection under 35 U.S.C. § 103 be reversed and that claims 13, 15, 16 and 18-24 be passed to issue.

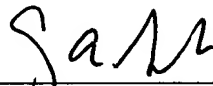
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document to **Deposit Account No. 03-1952** referencing docket No. 204372000301. However, the Assistant Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Respectfully submitted,

Dated: April 10, 2002

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## DETECTION AND CHARACTERIZATION OF THE PROSTATE-SPECIFIC MEMBRANE ANTIGEN (PSMA) IN TISSUE EXTRACTS AND BODY FLUIDS

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The prostate-specific membrane antigen (PSMA) glycoprotein is recognized by the monoclonal antibody (MAb) 7E11-C5.3 as a predominant 100 kDa and minor 180 kDa component in LNCaP cell line extracts and its expression has been shown by immunohistochemistry to be highly restricted to prostate epithelium. The aim of the present study was to utilize Western blot analysis to determine if PSMA could be detected in human tissue extracts and body fluids and if so, which molecular forms were present. PSMA was detected as 120 and 200 kDa bands in normal, benign and malignant prostate tissues and seminal plasma. Further analysis demonstrated that the larger molecular form of PSMA may be a dimer of the lower m.w. species. The PSMA glycoprotein was not detected in the majority of non-prostate tissue extracts examined except for a low yet significant amount in normal salivary gland, brain and small intestine, suggesting that PSMA may not be as prostate-specific as originally thought. Since the prostate-specific antigen (PSA) has been shown to be maximally shed into the serum in high-grade and metastatic prostate carcinomas, it was surprising that PSMA could not be detected in serum by Western blot analysis even in patients with actively progressive metastatic disease. Second generation antibodies generated against different epitopes may be required to determine if PSMA is shed into serum. Our results support the hypothesis that PSMA is a novel prostate biomarker.

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Prostate cancer (CaP) is the most common malignancy and the second leading cause of cancer death in males, with an estimated 244,000 new cases and 40,400 deaths in 1995 (Wingo *et al.*, 1995). As with any malignancy, early detection and treatment are paramount in reducing mortality in prostate carcinoma patients. The combined use of serum prostate-specific antigen (PSA) measurements and digital rectal examination have markedly enhanced the detection rate of prostate cancer. However, approximately 30% of benign prostatic hyperplasia (BPH) patients give false-positive PSA levels, and 25-30% of CaP patients have normal serum PSA concentrations (Cupp and Oesterling, 1993). These findings, and the recent controversy regarding whether and when to treat and what treatment modality to use, suggest that additional biomarkers need to be identified to differentiate benign from malignant prostate disease more accurately, to identify the clinically important prostate carcinomas and to develop potential targets for new treatment strategies.

A recently discovered prostate-associated biomarker designated prostate-specific membrane antigen (PSMA) may have the properties to meet some or all of these needs. This antigen was first described by Horoszewicz *et al.* (1987) using the mouse 7E11-C5 MAb produced against the LNCaP prostate carcinoma cell line. Immunohistochemical analysis showed that PSMA expression was highly restricted to normal, benign and malignant prostate epithelia (Horoszewicz *et al.*, 1987; Lopes *et al.*, 1990), and the physical nature of the antigen was initially identified in our laboratory as a predominantly 100 kDa membrane-associated glycoprotein (Abdel-Nabi *et al.*, 1992). The cDNA encoding the 100 kDa PSMA has been cloned and the DNA and amino acid sequence determined (Israeli *et al.*, 1993). Clinical trials using an <sup>111</sup>Indium-labeled conjugated form of MAb 7E11-C5.3 (<sup>111</sup>In CYT-356) to localize metastatic prostate carcinoma and sites of recurrence

following radical prostatectomy (Babaian *et al.*, 1994) were found to be superior to traditional staging and imaging modalities. Phase I clinical trials have been initiated to evaluate the efficacy of MAb 7E11-C5.3 radionuclide immunoconjugates for treating metastatic prostate cancer (Axelrod *et al.*, 1992). Another possible clinical application using RT-PCR to detect metastatic cells expressing PSMA mRNA in the whole blood of patients has been reported (Israeli *et al.*, 1995).

The PSMA glycoprotein appears to be an important new clinical biomarker of prostate cancer, yet no reports have critically evaluated its tissue specificity and examined whether it is shed into body fluids. Western blot analysis was used in the present study to determine conclusively if the PSMA glycoprotein could be detected with MAb 7E11-C5.3 in body fluids and tissue extracts from normal, benign and malignant prostates and to assess further the specificity of MAb 7E11-C5.3 by evaluating extracts from a variety of non-prostate tissues.

### MATERIAL AND METHODS

#### Cells and reagents

LNCaP and PC3 cells were obtained from the ATCC (Rockville, MD). DU145 cells were kindly provided by D. Mickey (Duke University). Cells were grown in RPMI 1640 supplemented with L-glutamine and 5% FCS (GIBCO-BRL, Gaithersburg, MD). The MAb 7E11-C5.3, purified by protein-A affinity chromatography from murine ascites, was provided by Cytogen (Princeton, NJ). The MAb concentration was determined using a single radial immunodiffusion system (TAGO, Burlingame, CA). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

#### LNCaP xenograft tumors

Male athymic (nu/nu) Swiss background nude mice and Fox Chase SCID inbred mice (C.B-17/ICrTac<sup>-</sup>acid DF), 4-6 weeks old (Taconic Farms, Germantown, NY) were housed in sterilized cages with filter bonnets and given autoclaved laboratory rodent chow (Purina, St. Louis, MO) and filtered tap water *ad libitum*. Mice were given injections of 2.5 mg of cyclophosphamide i.p. 1 day prior to s.c. injections in the left rear flank with  $1 \times 10^7$  LNCaP cells in exponential growth phase, in 0.2 ml of sterile medium or PBS. Subsequently, LNCaP tumors were propagated by s.c. implantation of tumor fragments aseptically transferred from donor to cyclophosphamide-treated, recipient mice. The same methods were used to grow LNCaP tumors in SCID mice, except SCID mice were not treated with cyclophosphamide.

#### Tissues and seminal plasma

Frozen human tissues, procured at the time of surgery or at autopsy were obtained from the Virginia Prostate Center tissue bank or through the Southeastern Cooperative Tissue Network (Birmingham, AL). All normal tissues were procured

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at autopsy, and benign and carcinoma tissues were obtained following surgery. Normal semen specimens were obtained from the Andrology Laboratory at the Howard and Georgianna Jones Institute for Reproductive Medicine, Eastern Virginia Medical School, following routine semen analysis. Semen from BPH and prostate carcinoma patients was obtained by masturbation, after informed consent.

Following collection, the semen samples were treated as previously described (Edwards *et al.*, 1981; Pulkkinen *et al.*, 1977), with minor modifications. Briefly, the samples were frozen prior to liquefaction and stored at  $-70^{\circ}\text{C}$ , until further analysis. The samples were thawed at room temperature by adding  $\frac{1}{2}$  volume dilution buffer (123 mM NaCl, 5 mM KCl, 1 mM  $\text{MgSO}_4$ , 1 mM EDTA and 37 mM Tris, pH 8.0). Pefablock (Boehringer Mannheim, Indianapolis, IN) was added to a final concentration of 0.1 mM and 50 $\times$  protease inhibitor cocktail (0.28 mM ANTI-PAIN; 0.75 mM PEPSTATIN; 60 mM EDTA) was added to a final concentration of 1 $\times$ . The samples were centrifuged for 5 min at 25,000g to remove cells or cellular debris and the supernatants, termed seminal plasma, were transferred to Eppendorf tubes and stored at  $-70^{\circ}\text{C}$ .

#### Membrane preparations

LNCaP cells were harvested and pelleted by centrifugation at 1,000g. The pellet was washed once with ice-cold PBS and pelleted again. The pellet was resuspended in hypotonic buffer (1 mM  $\text{NaCO}_3$ ) containing a protease inhibitor cocktail (as described above) and incubated on ice for 30 min; the cells were then lysed by Dounce homogenization. Surgical human tissue or murine xenograft tumors were prepared by mincing the tissue with scissors in 10 ml of hypotonic buffer containing protease inhibitor cocktail and homogenized using a Polytron homogenizer (Brinkmann, Westbury, NY).

The homogenates were centrifuged at 2,000g for 5 min in a Beckman JA20 rotor to pellet whole cells and nuclei. The supernatant was collected and centrifuged at 138,000g for 2 hr in a Beckman T150 rotor. The supernatant was discarded and the pellet, representing a crude membrane preparation, was either resuspended in PBS and stored at  $-70^{\circ}\text{C}$  or used for antigen purification.

#### Western blot analysis

Samples were loaded in equal protein concentrations (50  $\mu\text{g}/\text{lane}$  for membrane preps and 200  $\mu\text{g}/\text{lane}$  for seminal plasma) into the lanes of 4–20% gradient SDS-PAGE gels and blotted to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were incubated for 1 hr at  $37^{\circ}\text{C}$  in blocking buffer (5 $\times$  Denhardt's buffer, 1 $\times$  BBS, 0.1% NP-40 and 1.5% BSA). The blocking buffer was removed and the membranes incubated with 7E11-CS.3 (20  $\mu\text{g}/\text{ml}$ ) for 1 hr at room temperature. The primary antibody was removed and the membranes washed for 10 min  $\times$  3 with wash buffer (30 mM Tris, pH 7.5, 0.5 M NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS and 0.5 mM DTT); then membranes were incubated with horse anti-mouse horseradish peroxidase-labeled secondary antibody diluted in blocking buffer (1:10,000; Vector, Burlingame, CA) for 1 hr at room temperature. The secondary antibody was removed and the membranes washed for 10 min  $\times$  3 in wash buffer. The blots were developed using the ECL method (Amersham, Arlington Heights, IL) according to the manufacturer's instructions and exposed to X-ray film. Apparent m.w. were calculated on multiple blots using m.w. markers.

#### Competitive peptide blocking experiments

The competitive blocking studies were carried out like the Western blot experiments described above except the PSMA active peptide N1.19 (Troyer *et al.*, 1995) was added in a 20-fold molar excess over the MAb 7E11-CS.3 during the primary antibody incubation.

#### PSMA affinity column purification

Membrane preparations were resuspended in solubilization buffer (30 mM Tris, pH 7.5, 0.5 M NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS and 0.5 mM DTT) and protease inhibitor cocktail (as described above) and incubated for 2 hr at  $4^{\circ}\text{C}$  on a rotator. The solubilized material was centrifuged at 100,000g for 1 hr at  $4^{\circ}\text{C}$  to pellet non-solubilized material. The supernatant was collected, diluted 1:2 with 20 mM Tris, pH 8.0 and loaded onto an Affinica Protein A-7E11-CS affinity column constructed using the manufacturer's instructions (Schleicher and Schuell, Keene, NH). The column was washed with wash buffer (20 mM Tris, pH 8.0, 0.1% NP-40 and 0.1 mM DTT) and eluted with 2 N  $\text{NH}_4\text{OH}$ , pH 11.0. The eluted fraction was placed in 3,500 m.w. cut-off dialysis tubing (Spectrum, Houston, TX) and dialyzed against 20 mM Tris HCL, pH 4.0, containing 0.1 mM DTT for 2 hr at  $4^{\circ}\text{C}$ . The eluant was then concentrated against polyethylene glycol compound (m.w. 15,000–20,000) to approximately 500  $\mu\text{l}$ . Protein concentrations were estimated using the BCA protein assay following the manufacturer's instructions (Pierce, Rockford IL).

#### SDS-PAGE purification of PSMA

Affinity column-purified PSMA was loaded into the lanes of an SDS-PAGE mini-gel at 30  $\mu\text{g}/\text{lane}$ . One lane of purified PSMA and one m.w. marker lane were stained with Coomassie blue. The remaining PSMA bands were excised using the stained lane as a guide and placed in dialysis tubing (12–14,000 m.w. cut-off; Spectrum) containing 500  $\mu\text{l}$  CAPS buffer (10 mM CAPS, pH 11.0, 0.5 mM DTT) and electroeluted for 2 hr at 12 mAmp in CAPS buffer. The acrylamide bands were removed from the dialysis tubing and the eluted protein was dialyzed against distilled deionized water for 2 hr, then removed from the dialysis tubing and dried in a Savant (Farmingdale, NY) Speed-Vac concentrator.

#### Analysis of PSMA expression in serum

After informed consent, serum samples from normal donors were collected. Serum from patients with BPH or CaP were obtained from the tissue bank of the Virginia Prostate Center, Eastern Virginia Medical School. Serum samples from 5 non-pregnant normal women, 5 normal men under the age of 40, 5 BPH patients and 5 patients with stage D2 CaP were pooled. Twenty micrograms of affinity-purified PSMA was used to spike 500  $\mu\text{l}$  of the stage D2 pooled serum and incubated for 12 hr at  $37^{\circ}\text{C}$ . An additional 500  $\mu\text{l}$  of the same stage D2 pooled serum representing 29.09 mg of total protein was incubated in solubilization buffer (as described above) for 2 hr at  $4^{\circ}\text{C}$ , applied to a 7E11-CS.3 affinity column, eluted and prepared as described above. Twenty five micrograms of membrane preparations from LNCaP cells and normal prostate tissue; 100  $\mu\text{g}$  of a normal seminal plasma; 400  $\mu\text{g}$  of the spiked D2 serum; 20  $\mu\text{g}$  of affinity-purified stage D2 serum; and 400  $\mu\text{g}$  of non-spiked D2 serum, BPH serum, normal male serum and normal female serum were loaded into the lanes of 2 identical 4–20% SDS-PAGE gels, electrophoresed, blotted and developed for Western blot analysis as described above with either 7E11-CS.3 or an isotype-matched ( $\text{IgG}_1$ ) control antibody.

#### Two dimensional gel electrophoresis

Two-dimensional experiments were done using the Investigator 2-D Electrophoresis System (Millipore, Marlborough, MA) following the manufacturer's directions. Briefly, 10  $\mu\text{g}$  of affinity-purified antigen was loaded on top of a preparative isoelectric focusing tube gel and overlaid with sample overlay buffer. The tubes were electrophoresed for 17 hr at 1,000 Volts followed by 15 min, at 1,500 Volts. The gels were extruded onto pre-cast 4–20% gradient SDS-PAGE gels and layered with a 1% agarose sticker containing 0.1% bromophenol blue.

The second dimension was run at 16 mAmp overnight. The gel was removed from the apparatus and blotted to Immobilon-P (Millipore, Bedford, MA) for Western blot analysis. Isoelectric points were estimated by analyzing a tube gel, run at the same time as the sample, which contained only ampholytes. The analytical tube gel was cut into 1 cm sections and incubated in 3 ml double distilled water for 2 hr followed by pH measurement.

## RESULTS

### PSMA expression in prostate cell lines

Initial Western blots of membrane preparations from LNCaP xenograft tumors showed a predominant 100 kDa band and 2 higher m.w. species of approximately 180 and 160 kDa. Analysis of membrane extracts from cultured LNCaP cells gave identical results, whereas the prostate cell lines PC3 and DU145 did not express PSMA (Fig. 1 and data not shown). The 160 kDa band and an occasional 70 kDa band were transient and most likely represent breakdown products of the 2 major species. Furthermore, the 180 kDa band could be eliminated by increasing the SDS concentration in the sample buffer (data not shown).

### Two-dimensional gel electrophoretic analysis of PSMA

To characterize the PSMA expressed by the LNCaP cell line further, two-dimensional gel electrophoresis was performed using purified PSMA from *in vitro* cultured cell membrane preparations. Surprisingly, identical isoelectric points for both species were observed, with the major spots at isoelectric points of approximately 5.6, 5.7 and 5.8 for both the 100 and 180 kDa bands (Fig. 2). The tailing is consistent with the characteristics of integral membrane proteins. These results suggest that the 2 species are highly similar in their biochemical nature.

### Comparison of PSMA100 and PSMA180

The fact that identical isoelectric points were observed indicated that PSMA may exist in a dimer form. To look at this relationship more closely, affinity column-purified PSMA was separated by SDS-PAGE. The 2 m.w. species of 100 and 180 kDa are clearly evident (Fig. 3, lane 1) along with some smaller bands, which most likely represent breakdown products. The 2 major bands were excised, electroeluted, reappplied to an SDS gel and immunoblotted with MAb 7E11-CS.3. As shown in Figure 3, lane 2, the majority of the 100 kDa band remained at the appropriate m.w. However, a minority of this band migrated at the higher m.w. of 180 kDa. At the same time, the

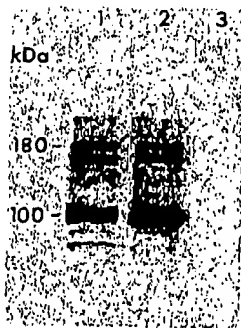


FIGURE 1 - Western blot analysis of 50 µg of membrane preparations from LNCaP nude mouse tumors (lane 1), cultured LNCaP cells (lane 2) and PC3 cells (lane 3). The 100 and 180 kDa bands are present in the LNCaP extracts but absent from PC3 membrane preparations.

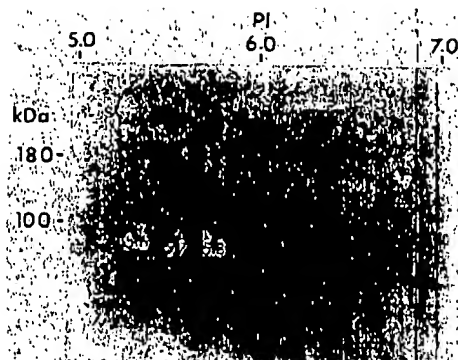


FIGURE 2 - Two-dimensional gel electrophoresis of affinity-purified PSMA. Ten micrograms of purified PSMA was analyzed as described in Material and Methods. Arrows indicate the isoelectric points of 5.6, 5.7 and 5.8 showing identical spots for both the 100 and 180 kDa species.

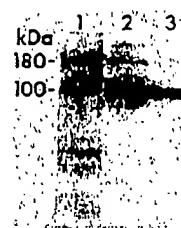


FIGURE 3 - Purified PSMA was separated on an SDS-PAGE gel and stained with Coomassie blue (lane 1); the predominant bands of 100 and 180 kDa and some smaller breakdown products are shown. The individual bands were excised and purified by electroelution from the gel slices. Gel-purified PSMA100 (lane 2) and PSMA180 (lane 3) were then reanalyzed by Western blot, which showed the majority of both species migrating at 100 kDa, indicating that PSMA180 is a dimer of PSMA100.

majority of the 180 kDa protein migrated faster at 100 kDa with a minority at 180 kDa (Fig. 3, lane 3). Increased concentrations of mercaptoethanol added to the loading buffer was not sufficient to reduce the 180 kDa band to 100 kDa in LNCaP membrane extracts, whereas increased SDS was able to denature the 180 kDa band totally to 100 kDa (data not shown). Additionally, trypsin digestion of the 2 bands followed by analysis on a silver-stained SDS-PAGE gel strongly indicated that the 2 bands were identical (data not shown). These data suggest that the 180 kDa species may be a dimer of the 100 kDa band or another unidentified molecule that may not be completely denatured by the normal concentration of SDS in the loading buffer used for the SDS-PAGE gel analysis.

### Detection of PSMA in prostate tissue

Membrane preparations of pathology confirmed normal, benign and malignant prostate tissues showed a Western blot banding pattern similar to that seen in LNCaP cell preparations except the migration of both the 100 and 180 kDa species had slightly slower mobilities of 120 and 200 kDa, respectively (Fig. 4a,b, arrows). This expression of PSMA was observed in cerebral cortex and salivary gland membrane extracts (Fig. 5, lanes 4 and 5). The size of PSMA expressed by the cerebral cortex was similar to that seen in LNCaP cell line extracts (approximately 100 kDa) whereas the size of PSMA detected in the salivary gland was similar to that seen in prostate



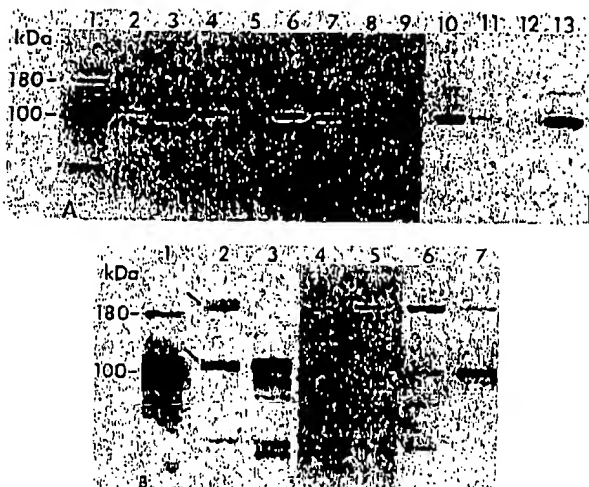


FIGURE 4 - Detection of PSMA in prostate tissue. (a) Western blot of 50 µg of LNCaP membrane preparation (lane 1), normal prostate tissue extracts (lanes 2-5), BPH tissue extracts (lanes 6-9) and CaP tissue extracts (lanes 10-13). (b) Western blot analysis of prostate extracts showing some of the heterogeneity seen in some samples—50 µg of LNCaP membrane preparation (lane 1), normal prostate tissue extracts (lanes 2 and 3), BPH tissue extracts (lanes 4 and 5) and CaP tissue extracts (lanes 6 and 7). Arrows indicate the shift in the size of the bands from the 100 and 180 kDa of LNCaP PSMA to the approximately 120 and 200 kDa of the PSMA seen in tissue extracts.

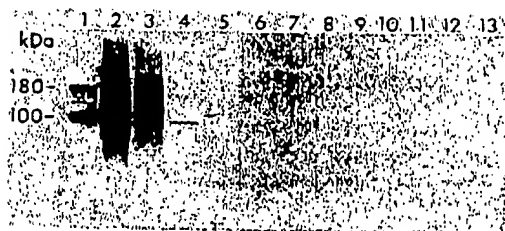


FIGURE 5 - Detection of PSMA in normal non-prostate tissue extracts. Western blot of 50 µg of membrane preparations from LNCaP cells (lane 1), small intestine (lanes 2 and 3), cerebral cortex (lane 4), salivary gland (lane 5), skeletal muscle (lane 6), cardiac muscle (lane 7), colon (lane 8), breast (lane 9), lung (lane 10), ovary (lane 11), kidney (lane 12) and liver (lane 13). Lane 3 represents 25 µg of the same small intestine shown in lane 2. The small intestine (lanes 2 and 3) shows a smearing with no detectable bands, similar to what is seen in prostate tissues. The PSMA in the cerebral cortex (lane 4) is similar in size to the 100 kDa species of LNCaP cells, whereas the PSMA expressed in salivary gland (lane 5) is similar to that seen in prostate tissue.

tissue extracts (approximately 120 kDa). The PSMA glycoprotein was not expressed in the majority of non-prostate tissues examined including normal skeletal and cardiac muscle, colon, breast, lung, ovary, kidney and liver tissues (Fig. 5, lanes 5-12) or a variety of non-prostate malignancies including colon, lung, bladder, liver and breast adenocarcinomas (Fig. 6, lanes 2-6).

#### 7E11-CS.3 reactive bands could be competitively blocked with PSMA peptides

The peptide epitope for MAb 7E11-CS.3 on the PSMA glycoprotein has been determined to be located at the amino terminal end of the polypeptide backbone (Troyer *et al.*, 1995).

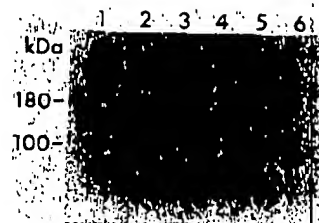


FIGURE 6 - Detection of PSMA in non-prostate malignancies. Western blot analysis of 50 µg of membrane preparations from LNCaP cells (lane 1), colon (lane 2), lung (lane 3), bladder (lane 4), liver (lane 5) and breast adenocarcinoma (lane 6) showing the typical 100 and 180 kDa bands in the LNCaP cells but no expression in other malignant tissues.

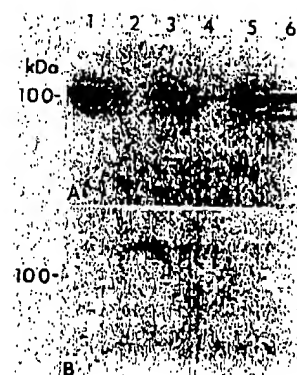
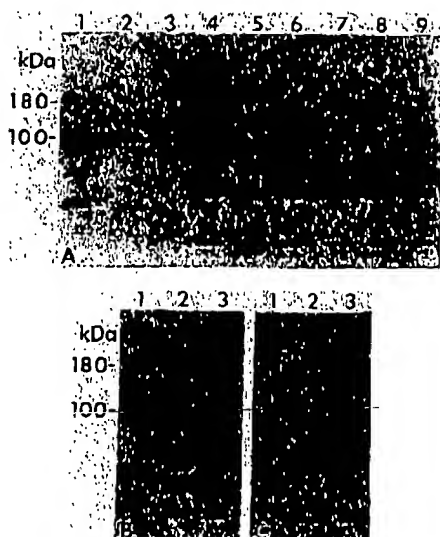


FIGURE 7 - Western blot competitive inhibition of MAb 7E11-CS.3 with the PSMA peptide N1.19. (a) Membrane extracts from LNCaP (lane 1), PC3 (lane 2), normal prostate (lane 3), benign prostate (lane 4), prostate carcinoma (lane 5) and a seminal plasma from a prostate carcinoma patient probed with MAb 7E11-CS.3. (b) The identical blot probed with MAb 7E11-CS.3 plus a 20 M excess of the PSMA N1.19 peptide. The N1.19 peptide is clearly able to abrogate MAb 7E11-CS.3 binding to its target antigen on prostate cell lines, tissues and seminal plasma.

The reactive peptide N1.19, representing the first 19 amino acids of PSMA, was used to block MAb 7E11-CS.3 reactivity competitively in immunoblot assays. The reactivity to LNCaP cell line extracts, prostate tissue extracts, and seminal plasma (Fig. 7a) could be totally eliminated by incubating the antibody with a 20-fold molar excess of the N1.19 peptide (Fig. 7b).

#### PSMA was not detected in the serum of prostate carcinoma patients

Since other prostate markers are detected in significant concentrations in serum, we wanted to determine if PSMA could also be detected in serum by Western blot analysis. Figure 8a shows the analysis of serum by Western blotting with MAb 7E11-CS.3. When these blots were exposed for the standard amount of time (1 to 2 min), no bands were evident, but when the blots were exposed for more than 10 min the pattern in Figure 8 was observed. Lanes 1-3 in Figure 8a represent LNCaP, normal prostate tissue membrane extracts and normal seminal plasma, respectively. Note the difference in size of the PSMA expressed in LNCaP (Fig. 8a, lane 1) compared with the tissue and seminal plasma (Fig. 8a, lanes 2 and 3). The size of PSMA shed into serum would be expected to be similar to the size of PSMA observed in tissue extracts and seminal plasma. Sera from 5 stage D2 CaP patients were pooled and analyzed in several ways. First, the serum was



**FIGURE 8**—Detection of PSMA in serum. (a) Western blot, using 7E11-C5.3, of 25 µg of an LNCaP membrane extract (lane 1) and normal prostate (lane 2) membrane extracts, 100 µg of normal seminal plasma (lane 3), 400 µg of spiked pooled stage D2 serum (lane 4), 20 µg of affinity-purified stage D2 serum (lane 5), 400 µg of pooled serum from stage D2 CaP (lane 6), BPH (lane 7), normal man (lane 8) and normal women (lane 9). (b) Western blot of 25 µg of an LNCaP membrane extract (lane 1), 400 µg of pooled D2 serum (lane 2) and 800 µg of pooled D2 serum blotted with MAb 7E11-C5.3. (c) The identical blot probed with MAb 7E11-C5.2 plus N1.19 PSMA peptide. No PSMA was detected by affinity chromatography of the pooled D2 serum (a, lane 5), and whereas bands are present in the pooled sera, the same bands are present in the pooled normal male and female serum. The bands in the LNCaP extract were eliminated by competition with the N1.19 peptide whereas the bands in the pooled serum remained.

spiked with affinity-purified LNCaP PSMA and incubated for 12 hr at 37°C prior to analysis by Western blot (Fig. 8a, lane 4). The PSMA in the spiked serum was at the expected m.w. of 100 and 180 kDa, like the PSMA seen in LNCaP extracts, along with a larger band of unknown composition. Second, 29 mg of the pooled stage D2 serum was solubilized, passed through a MAb 7E11-C5.3 affinity column, washed and eluted from the column to remove the large concentrations of albumin and other proteins that make Western blot analysis of serum difficult. No PSMA was detected in the affinity column eluant although a significant amount of serum proteins remained, particularly bands migrating at the expected m.w. of the heavy and light immunoglobulin chains, which may have adhered to the protein-A used to construct the affinity column (Fig. 8a, lane 5). Third, 400 µg of the same stage D2 serum and 400 µg of a pooled BPH, a pooled normal male and a pooled normal female serum (Fig. 8a, lanes 6–9) were analyzed. Several bands were present following overexposure in all the sera including the normal female serum but none at the expected m.w. of 120 kDa.

The bands present in the pooled stage D2 serum did appear to be overexpressed compared with the BPH and normal sera. The presence of identical bands in the pooled normal female serum suggested that the bands seen on this blot were non-specific. To determine if this were the case, the D2 pooled serum was subjected to SDS-PAGE gel electrophoresis in duplicate along with an LNCaP membrane extract. One of the blots was probed with MAb 7E11-C5.3 and the duplicate blot was probed with MAb 7E11-C5.3 containing the N1.19 PSMA

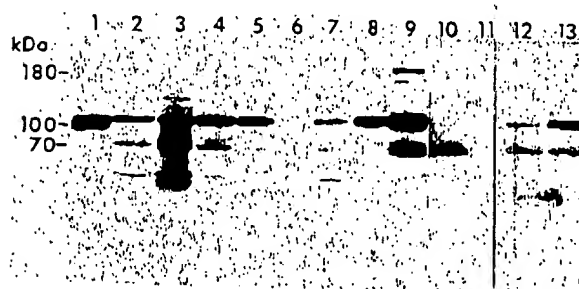
peptide. Figure 8b shows the banding pattern for the LNCaP extract (Fig. 8b, lane 1) and 400 and 800 µg of the pooled D2 serum (Fig. 8b, lanes 2 and 3, respectively). There is a reactive band at the approximate m.w. of 100 kDa in this pooled serum that could be interpreted to be PSMA. However, while all of the reactive bands seen in the LNCaP membrane extract were eliminated by the N1-19 peptide competition (Fig. 8c, lane 1) none of the bands in the serum were competed out (Fig. 8c, lanes 2 and 3). An identical pattern could also be produced if the pooled serum blot was probed with only the secondary antibody or with an isotype-matched IgG; control antibody in place of MAb 7E11-C5.3 (data not shown).

#### Detection of PSMA in seminal plasma

Since PSMA is a product of the prostate glandular epithelium, it was of interest to determine whether it could be detected in seminal plasma. Western blot analysis of seminal plasma obtained from normal (NSP), BPH (BSP) and prostate carcinoma patients (CaSP) showed that PSMA was readily detectable in seminal plasma with expression of the 120 kDa band and occasionally the 200 kDa band, similar to the pattern seen in tissue extracts (Fig. 9). NSP showed the most consistent expression of PSMA, with little variation from sample to sample, demonstrating the 120 kDa band and often an 80 kDa band (Fig. 9, lanes 2–5). BSP, like the BPH tissue extracts, exhibited variable expression of PSMA ranging from very low to overexpression (Fig. 9, lanes 6–9). The majority of the CaSP samples were similar in banding pattern to NSP, although one sample showed only the 180 kDa band. While it appears that the concentration of PSMA in CaSP is lower than in NSP, the total protein of these samples was significantly higher than in NSP or BSP. In fact, when equal volumes of samples instead of equal protein concentrations were used for the immunoblots, the intensities of the CaSP bands were comparable to those seen in the normal samples (data not shown).

#### DISCUSSION

PSMA is a new prostate biomarker that appears to be overexpressed in poorly differentiated and metastatic prostate carcinomas (Wright *et al.*, 1995). Antibody-radionuclide conjugates have been successfully used to localize metastatic disease *in vivo* (Babaian *et al.*, 1994) and to treat human prostate



**FIGURE 9**—Detection of PSMA in seminal plasma. Western blot of 50 µg LNCaP membrane preparation (lane 1) and equal protein concentrations (200 µg/lane) of normal seminal plasma (lanes 2–5), BPH seminal plasma (lanes 6–9) and CaP seminal plasma. Lanes 1–9 represent an exposure time of 2 min and lanes 10–13 represent an exposure of 5 min. Note the slightly larger m.w. of PSMA in the seminal plasma compared with LNCaP extracts. Normal seminal plasma shows a consistent expression of PSMA whereas BPH seminal plasma is quite variable. The expression of PSMA in CaP seminal plasma appears to be lower, which may result from the much higher protein concentrations seen in CaP compared with normal or BPH seminal plasma.

tumors in nude mice (Axelrod *et al.*, 1992). These reports suggest that PSMA may have important diagnostic and therapeutic applications. The present study provides information on the presence and molecular size characteristics of PSMA in body fluids and tissue extracts from normal donors and from patients with benign and malignant tumors.

The finding of 120 and 200 kDa bands in prostate tissue extracts was in contrast to the 100 and 180 kDa bands found in membrane extracts and purified PSMA preparations from the LNCaP prostate carcinoma cell line. This slower mobility of PSMA in tissue extracts and seminal plasma may result from a post-translational modification or an alternative splicing event. The fact that normal, BPH and CaP tissue extracts exhibited the same size components (either the 120 kDa band, the 200 kDa band or both) indicates that the PSMA glycoprotein recognized by Mab 7E11-C5.3 is synthesized identically in normal and pathological prostate tissues. Additionally, the detection of PSMA in normal prostate tissue did not appear to be influenced by the significant time period between death and the collection of tissue during autopsy since the normal prostatic tissue (autopsy specimens) and CaP tissue (surgical specimens) showed similar results.

The occasional higher m.w. species was most often seen when there was a large amount of the smaller band, suggesting that a strong relationship may exist between the low and high m.w. components. Although it is possible that the 100/120 kDa species was a breakdown product of the 180/200 kDa band or that the 180/200 kDa component represented a precursor molecule of the 100/120 kDa band, one- and two-dimensional electrophoretic analysis of purified PSMA from LNCaP extracts demonstrated that the 180 kDa species may be a dimer of the 100 kDa component. The findings that the 100 kDa band may redimerize following purification, a phenomenon that has been reported to occur with other proteins (Huth *et al.*, 1993), that the dimer was observed under denaturing and reducing conditions (Reiser *et al.*, 1992) and that the PSMA cDNA clone encodes for a 100 kDa glycoprotein (Israeli *et al.*, 1994) support the hypothesis that the 180/200 kDa component is most likely a dimer of 2 PSMA 100/120 kDa molecules.

PSMA was not found in extracts of other prostatic cell lines, supporting previous reports (Horoszewicz *et al.*, 1987; Israeli *et al.*, 1994). Horoszewicz *et al.* (1987) reported the finding of PSMA in frozen sections of proximal tubules of normal kidneys, and Lopes *et al.* (1990) described PSMA expression in cardiac and skeletal muscle by immunoperoxidase staining. The same immunostaining reactivity was observed in formalin-fixed, paraffin-embedded sections of these normal tissues (data not shown). However, in the present study, PSMA was not detected in extracts of these same normal tissues by Western blot analysis, which suggests that the immunoperoxidase staining was non-specific. This conclusion is consistent with the fact that 7E11-C5.3 antibody-isotope conjugates did not localize to skeletal muscle in mice (Lopes *et al.*, 1990) or in monkeys (D. Lopes, Princeton, NJ, personal communication). The detection of PSMA in extracts of normal cerebral cortex of the brain, normal salivary gland and normal small intestine correlates with the finding of PSMA mRNA in brain and salivary gland and no mRNA in other normal tissues, including skeletal muscle (Israeli *et al.*, 1994). It is of interest to note that PSA has been found to be present in breast, colon, ovarian, parotid, kidney and liver tumors, normal breast, amniotic fluid, breast milk (Levesque *et al.*, 1995), normal salivary gland (van Krieken, 1994) and normal endometrium (Clements and Mukhtar, 1994). These results suggest that PSA as well can no longer be regarded as a specific biomarker of the prostate gland. In spite of the detection of PSMA in these tissues by immunoblotting, it has not been detected by immunostaining of frozen sections (Horoszewicz *et al.*, 1987; Lopes *et al.*, 1990)

or paraffin-embedded sections (data not shown) of normal brain, salivary gland and small intestine. This disparity may be the result of masked antigenic epitopes in the tissue sections or the fact that expression was below the detection limits of the immunohistochemistry assay. By using more sensitive assays, PSMA, like PSA, may eventually be found to be present in a variety of human tissues. The possibility that PSA may have a broader function than its association with semen liquefaction (Levesque *et al.*, 1995) points to the importance of determining the function of PSMA.

The finding that different species of PSMA were detected in brain (100 kDa) and salivary gland (120 kDa) supports the hypothesis that the differences seen between PSMA from LNCaP cells and prostate tissue extracts may result from a change in post-translational modification or a splicing variant and are not artifacts of *in vitro* cell culture. The high m.w. smearing and multiple banding pattern of the immunoblot of the small intestine formed a pattern markedly different from that observed in LNCaP cell extracts and prostate and non-prostate extracts. The banding pattern was suggestive of a heavily glycosylated glycoprotein or mucin. It is quite possible that Mab 7E11-C5.3 binds to an epitope other than PSMA in the small intestine, or such a pattern may be due to non-specific binding of the antibody. Since the secondary antibody (used without the primary Mab 7E11-C5.3) did not bind blots of small intestine, it appears likely that Mab 7E11-C5.3 specifically bound to a component in this extract. PSMA may be glycosylated differently in the intestine, thereby resulting in the different banding pattern.

The detection of PSMA in seminal plasma but not serum by immunoblot analysis was surprising since previous reports suggested that PSMA could be detected in serum by an immunoassay (Horoszewicz *et al.*, 1987) and Western blot (Rochon *et al.*, 1994). The analysis of serum in the present study resulted in multiple bands similar to those reported by Rochon *et al.* (1994). They identified a band of 116 kDa as the PSMA component. In the present study a band between 100 and 120 kDa was observed, and although it did appear that the bands in pooled stage D2 serum were slightly overexpressed compared with normal and BPH serum, the same banding pattern was observed in both female and male serum, suggesting that these bands were the results of non-specific binding with either the primary or secondary antibody. When the PSMA peptide (N1.19) containing the Mab 7E11-C5.3 epitope (Troyer *et al.*, 1995) was incubated with the antibody prior to use as a probe, the PSMA bands in the LNCaP cell extract were eliminated, whereas all the bands in the serum samples remained. These results clearly suggest that the bands observed in serum are non-specific, probably the result of non-specific binding by the secondary antibody. Since Rochon *et al.* (1994) did not compete the reactivity with purified PSMA or show results using only the secondary antibody, the 116 kDa band they reported as being PSMA is most likely a serum protein reacting with the secondary antibody. In fact, we could demonstrate an identical banding pattern in serum using only the secondary antibody for the immunoblot (data not shown). These results and conclusions do not negate the possibility that PSMA is shed into serum. The failure to detect PSMA by Western blot may have been the result of degradation or masking of the antigenic epitope, although purified PSMA or specific PSMA peptides added to serum were found to be stable and of the correct m.w. when immunoblotted. Alternatively, the concentration of PSMA in serum may have been too low to be detected by Western blotting. However, PSMA was not detected following the enrichment (affinity purification) of serum over 200-fold.

The 120 or the 200 kDa band (or both) PSMA components were found to be present in seminal plasma from normal

donors and from patients with benign or malignant prostate tumors. The PSMA detected in seminal plasma from normal males and prostate carcinoma patients showed a consistent banding pattern, whereas the banding pattern in seminal plasma from BPH patients varied greatly. This variation is consistent with the observed reduction in PSMA mRNA (Israeli *et al.*, 1994) and PSMA antigen expression (Wright *et al.*, 1995) in BPH specimens. The often low, absent and variable expression of the 120 kDa band in the BPH specimens may have resulted from splicing variants or post-translational modifications.

Previously we described the mapping of the MAb 7E11-C5.3 epitope on PSMA (Troyer *et al.*, 1995) and analyzed PSMA in prostate tissue immunohistochemically (Wright *et al.*, 1995). In the present study we have shown that PSMA exists in tissues and seminal plasma as a predominant 120 kDa band; by contrast, PSMA is found as a 100 kDa glycoprotein in extracts of LNCaP cells. Occasionally, a dimer form of PSMA having a m.w. of 180 (LNCaP cell extracts) or 200 kDa (tissue extracts, seminal plasma) is observed. The finding of PSMA in seminal plasma, coupled with the overexpression of PSMA observed in poorly differentiated and metastatic prostate tissues (Wright *et al.*, 1995), suggests that measurement of PSMA concentrations

in seminal plasma might be used to predict and monitor tumor progression. Although Western blot analysis failed to detect PSMA in serum, further studies will be required to determine if any form of PSMA is shed in serum. The production of second generation antibodies against different antigenic epitopes other than the one recognized by MAb 7E11-C5.3 may be required to develop a highly sensitive immunoassay to determine if PSMA is or is not present in serum. Finally, the finding of PSMA in extracts of brain, salivary gland and small intestine raises certain issues regarding the safety of using MAb 7E11-C5.3 immunoconjugates for diagnostic imaging and especially antibody-targeted therapy. These cross-reactivities, however, should not dampen efforts to understand fully the function and clinical potential this novel biomarker may have in the diagnosis and therapy of prostate cancer.

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**APPENDIX A**

**Currently Pending Claims**

13. A method to elicit an antitumor immune response to prostate tumors in a subject, which method comprises

administering to said subject at least one active ingredient formulated for administration to said subject,

wherein said active ingredient is human prostate-specific membrane antigen (PSMA); or prostatic acid phosphatase (PAP); or mixtures of the foregoing; or

is a nucleic acid that generates PSMA or PAP, or mixtures of PSMA and PAP *in situ*.

15. The method of claim 13 wherein said active ingredient is human PSMA.

16. The method of claim 13 wherein said active ingredient is PAP.

18. The method of claim 13 wherein said active ingredient is a nucleic acid that generates PSMA *in situ*.

19. The method of claim 13 wherein said active ingredient is a nucleic acid that generates said PAP *in situ*.

20. The method of claim 13 wherein the active ingredient is encapsulated in liposomes and/or coupled to liposomes.

21. The method of claim 20 wherein said liposomes contain an adjuvant.

22. The method of claim 13 which further includes at least one adjuvant that enhances the antitumor immune response.

23. The method of claim 22 wherein said adjuvant is selected from the group consisting of Freund's complete adjuvant; alum; lipid A; monophosphoryl lipid A; *Bacillus Calmette-Guerin* (BCG) or other bacteria polysaccharides; saponins; detoxified endotoxin

(DETOX); muramyl tripeptide or muramyl dipeptide or their derivatives; SAF1; lymphokines; cytokines; colony stimulating factors; nonionic block copolymers; and immune stimulating complexes (ISCOMS).

24. The method of claim 13 wherein said subject is afflicted with metastatic prostate cancer; and/or wherein said subject has been surgically treated to excise said tumor but is at risk for recurrence.

### **Tumor-associated antigen:**

A molecule specifically or preferentially expressed by tumoral cells, but not, or hardly, by normal cells. Such molecules can be used as vaccination targets to destroy the tumor.

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## **APPENDIX B**

Last Update: Apr 29, 1996

## Acid phosphatase

### Structure/function relationships in acid phosphatases

Prostatic acid phosphatase is found in high concentrations in the prostate and the seminal fluid. The enzyme is used as a marker in the diagnosis of prostate cancer, since the development of metastatic prostate cancer is accompanied with an increase of acid phosphatase activity in the blood. The biological substrate and the physiological function of this enzyme is not known. We have determined the crystal structure of recombinant unliganded rat prostatic acid phosphatase and of complexes of the enzyme with the inhibitor tartrate and the transition state analogues vanadate and molybdate, respectively.

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### APPENDIX C